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Leptin cDNA cloning and its mRNA expression in plateau pikas (*Ochotona curzoniae*) from different altitudes on Qinghai-Tibet Plateau

J. Yang [a,b], X.Q. Zhao [a,*], S.C. Guo [a,b], H.G. Li [a,b], D.L. Qi [a,b], D.P. Wang [a,b], J.H. Cao [a,b]

[a] *Northwest Plateau Institute of Biology, The Chinese Academy of Sciences, Xining 810001, PR China*
[b] *Graduate School of the Chinese Academy of Sciences, Beijing 100039, PR China*

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Abstract

Leptin, an adipocyte-derived hormone, plays an important role in body energy homeostasis. Plateau pika (*Ochotona curzoniae*), an endemic and keystone species living only at 3000–5000 m above sea level on Qinghai-Tibet Plateau, is a typically high hypoxia and low temperature tolerant mammal with high resting metabolic rate (RMR), non-shivering thermogenesis (NST), and high ratio of oxygen utilization to cope with harsh plateau environment. To explore the molecular mechanism of ecological acclimation in plateau pika, we first cloned pika leptin cDNA and compared its mRNA expression in different altitudes (3200 and 3900 m) using real-time RT-PCR (Taqman probe) technology. The full-length pika leptin cDNA was 3015 with 504 bp open-reading frame encoding the precursor peptide of 167 amino acids including 21 residues of signal peptide. Pika leptin was 70–72% homologous to that of other species and was of similarly structural characteristics with other species. The pika-specific genetic diversity in leptin sequence occurred at twenty sites. With the increase in altitude, there were larger fat store and high level of ob gene expression in plateau pika. Our results indicated that leptin is sensitive to cold and hypoxia plateau environment and may play one of important roles in pika's ecological adaptation to harsh plateau environment.

Keywords: Leptin; cDNA cloning; Plateau pika; mRNA expression in different altitudes

Leptin, the product of ob gene, is a 16 kDa cytokine-like hormone and mainly secreted from adipose tissue [1]. Leptin, acting on its receptors in hypothalamus and other several peripheral tissues, exerts diverse biological effects, including regulation of energy homeostasis, glucose metabolism, lipid oxidation, reproduction, blood pressure, hematopoiesis, angiogenesis, brain and bone development, wound healing, and cell differentiation and proliferation [2]. Leptin expression and secretion is influenced by many factors. Leptin synthesis is related with adipocyte size, for larger adipocytes containing more leptin than small ones in the same individual [3]. The level of leptin in serum correlates with total body fat stores [4,5]. The changes in leptin expression in response to fasting and feeding are mediated by insulin. Fasting can inhibit leptin expression, while after feeding leptin synthesis is increased [6,7]. Due to sex differences in body fat distribution and testosterone level, females have higher leptin level than males when matched by age, weight, and body fat [8,9]. Hypoxia induces the increase in leptin expression [10,11]. Cold exposure can lead to reduction of leptin expression by directly acting on adipocyte or indirectly being mediated by the sympathetic nervous system [12,13].

All organisms face and respond to variation in their environments and deal with these environmental stresses by metabolic adjustments, then eventually reach stable status in adaptation to their environment [14]. Qinghai-Tibet Plateau, more than 3000 m above sea level on average, is the highest and largest plateau in the world and possesses

* Corresponding author. Fax: +86 971 6143282.
E-mail address: xqzhao@nwipb.ac.cn (X.Q. Zhao).

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unique environment of nature and geography. Strong ultraviolet radiation, hypoxia, and cold environment are the most obvious climate characters on plateau, which have profound effects on animal survival. This unique climate has made Qinghai-Tibet Plateau become a natural laboratory for the research in hypoxia and cold adaptation. In long-term evolution, many native animals have formed their own unique mechanism in adaptation to harsh plateau environment, and produced some important characters which help organism keep away from unfavorable environment and effectively acquire matter or energy from their environment to ensure their normal growth and reproduction. Plateau pika (*Ochotona curzoniae*), an endemic species of Qinghai-Tibet Plateau, also called ''black-lipped pika'', is a small, non-hibernation, diurnal lagomorph that inhabits alpine meadows on the Qinghai-Tibet Plateau, in China. The animal is sexually monomorphic in size and even has little difference in external sexual anatomy. Plateau pika is known as a keystone species on Qinghai-Tibet Plateau ecosystem and plays an important role in preservation of native biodiversity [15–18]. In evolution, pika has become a high hypoxia and low temperature tolerant mammal with markedly high resting metabolic rate (RMR), non-shivering thermogenesis (NST), and high ratio of oxygen utilization to cope with cold and hypoxia plateau environment [19–21]. Therefore, plateau pika has become a typical mammal for the research in cold and hypoxia adaptation to plateau environment.

Due to apparent changes of energy metabolism in plateau pika, and diversified function of leptin, especially in regulation of energy homeostasis, and expression regulation of leptin, we hypothesized that leptin may be play important roles in pika's ecological mechanism in adaptation to harsh plateau environment. Therefore, to reveal the effects of leptin on ecological acclimation of plateau pika, it is necessary for us to study its primary structure and function. In this work, we first cloned plateau pika leptin cDNA using the methods of RT-PCR and RACE, and depicted expression pattern of ob mRNA in plateau pikas from different altitudes using real-time PCR technology. The aims of this study were: (1) to characterize the leptin cDNA of plateau pika, (2) to reveal leptin expression character in plateau pikas from different altitudes, (3) to estimate whether leptin expression characters are consistent with physiological changes of plateau pika in high altitude, and (4) to evaluate its ecological significance for plateau pika in adaptation to harsh plateau environment.

Materials and methods

Experimental sites, animal sampling. The experimental animals selected were from two sites: Haibei alpine meadow ecosystem research station (lat 37°29′N,long 101°12′E) and Dawu town, Guoluo Maqing, in Qinghai province ((lat 37°25′N, long 100°30′E), in China. The two sites are located on the typical alpine meadow. The altitudes at the two sites are 3200 and 3900 m, respectively. The annual mean air temperatures recorded in the last decade in the two sites are −1.7 and −2.6 °C, respectively. There is no absolute frost-free period during any part of the year at the two sites. The annual average growth period of herbage in these two regions is 110–130 days. There are two main vegetation types in those two regions, alpine meadow and alpine shrub. Climate data of Haibei referred the data of Haibei alpine meadow ecosystem research station (not shown in the present study). Data for Guoluo referred to Wang et al. [22].

Sampling was performed at time between 10:00 am and 3:00 pm in August. We delimited a dimension of 200 m × 200 m as a quadrat in each altitude region. All animals were live-trapped in each quadrat at random. After measuring body weight and body length, and identifying hair color and gender, we chose male plateau pikas as objects of study. The sample size in each altitude was ten.

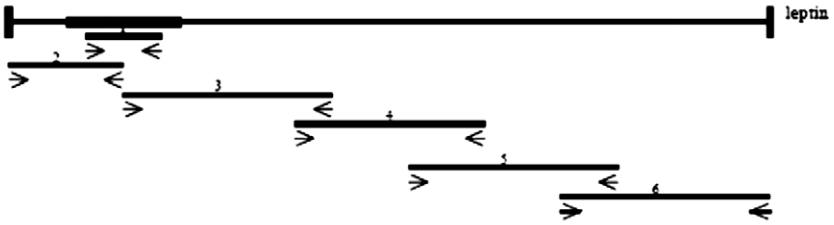
The pikas were first anesthetized with chloral hydrate (5%), and then killed by cervical dislocation and dissected on the spot. The White adipose tissues (WAT) at abdominal subcutaneous were immediately frozen in liquid nitrogen and then stored at −70 °C until use. All procedures involved in the handling and care of animals were in accordance with the China Practice for the Care and Use of Laboratory animals and were approved by China Zoological Society.

Cloning strategy and primers design. To obtain complete cDNA of plateau pika leptin, six individual fragments (OCLEP, OCLEPA, OCLEPB, OCLEPC, OC5R, and OC3R), which were overlapping in partial region between each two neighboring fragments, were cloned in turns. The procedures of cloning were described as follows: The fragment (OCLEP) within the coding region of leptin cDNA was first amplified. The forward and reverse primers (LEPf and LEPr) for OCLEP were designed from the alignment of highly conserved coding sequence regions of ob gene of Mus musculus (Accession No. NM_008493), Homo sapiens (Accession No. NM_000230), Bos Taurus (Accession No. BT020625), Sus scrofa (Accession No. NM_213840). Subsequently, other three fragments (OCLEPA, OCLEPB and OCLEPC) were cloned in order. The pika leptin-specific forward primer was designed from the sequence of neighboring fragment cloned previously; the reverse primer for each fragment was designed from the alignment of conversed sequence in 3′ untranslated regions of leptin cDNA of above species. The fragments (OC5R and OC3R) were attained by 5′ and 3′-rapid amplification reaction (3′ and 5′ RACE). In these six fragments, a 70–100 bp overlapping region was formed between each two neighboring fragments. The cloning strategy and primers used for PCR are shown in Fig. 1.

The cloning of leptin cDNA of plateau pika. Total RNA was isolated from abdominal subcutaneous adipose tissue using TRIzol reagent (Invitrogen, USA) and treated with RNase-free DNase I (TaKaRa Biotechnology Co., Ltd). RT-PCR was performed with Access RT-PCR System (Promega, USA). The 5′ and 3′-rapid amplification reaction (5′ and 3′ RACE PCR) was performed with SMART RACE cDNA Amplification kit (Clontech, USA) and Advantage 2 PCR Enzyme Mix kit (Clontech, USA). All of above procedures were done according to the corresponding manufacturer's instructions. The target DNA fragments of expected size were purified and subcloned into PGEM-T Easy Vector (Promega, USA), then sequenced. The full-length leptin cDNA of plateau pika was spliced and determined according to the overlapping regions formed in these fragments. Then the entire sequence was submitted into GenBank database.

Sequence analysis. Translation of cDNA nucleotide sequence was performed using the EditSeq program of DNASTAR. The nucleotide and deduced amino sequence were compared with the sequences in the GenBank database using BLAST program available for the NCBI internet website (http://www.ncbi.nlm.nih.gov.). The signal peptide in the deduced amino acid sequence was predicted with the tool of SignalP (http://www.cbs.dtu.dk/services/signalP). Multiple alignment was done by the program CLUSTALX (1.81). The functional motifs were predicted in the deduced amino acid sequence using the tool of MotifScan program in PROSITE database of protein families and domains (http://www.expasy.org/prosite). The secondary and tertiary protein structures were estimated at the SWISS-MODEL automated comparative protein modelingserver (http://www.expasy. org/swissmod/SWISS-MODEL.html) based upon human leptin (1AX8.pdb) Protein Data Bank (PDB) structure file tocompare structural similarities of human and plateau pika.

A Primers and location of expected fragments



B Primer sequence for PCR amplification

clone name	primer name	primer type	application	Sequences (5'-3')
OCLEP	LEPf	forward	RT-PCR	CCT CAT CAA GAC CAT TGT CAC
	LEPr	reverse	RT-PCR	TGC TCA AAG CCA CCA CCT
OCLEPA	LEPAf	forward	RT-PCR	AAA AGT CCA GGA TGA CAC CA
	LEPAr	reverse	RT-PCR	CCA GAA TAA AAC GCA TAA TAA AT
OCLEPB	LEPBf	forward	RT-PCR	TCT GGG GAA GCG TGT CTT GAA GG
	LEPBr	reverse	RT-PCR	CCT GCT CTG GGG ATC ACC ACC T
OCLEPC	LEPCf	forward	RT-PCR	GGT TCA GAA TGG ATT TCC TAA GT
	LEPCr	reverse	RT-PCR	GCT TCA AAG GGA TGT GGC
OC5R	OC5r	reverse	5'RACE-PCR	GGG AAG GCA GAC TGG TGA GGA T
OC3R	OC3f	forward	3'RACE-PCR	TGG CAT GTG CAT ACT TTC AGG ACA

Fig. 1. Cloning strategy and primers used for amplification. (A) Schematic representation of the structure and sequencing strategy for plateau pika leptin cDNA. The open-reading frame is depicted by closed boxes. PCR products (solid bar) are indicated. The two arrows in each PCR product indicate the forward and reverse primers. (1, OCLEP; 2, OC5R; 3, OCLEPA; 4, OCLEPB; 5, OCLEPC; and 6, OC3R). (B) Primer sequences used for PCR.

Primers and TaqmanTM probes for real-time PCR. The primers and probes for the *ob* gene and endogenous *β-actin* gene were designed using the program Primer Express™ (Perkin-Elmer, Applied Biosystems, USA) following the recommended criteria. The forward primer for *ob* gene (270F: 5′-CCC GGA ATG TGG TCC AAA-3′) and the reverse primer (335R: 5′-CAG CTA CCA GGT GCA GAA GGT-3′) amplified an 86 bp fragment. The sequence of probe for *ob* gene was 289T: 5′-TGC CAA TGA CCT GGA GAA CCT CCG-3′. The forward primer for *β-actin* gene (BT277F: 5′-GCG AGA TCG TGC GTG ACA T-3′) and the reverse primer (BT343R: 5′-GCC ATC TCC TGC TCG AAG TC-3′) amplified an 86 bp fragment. The sequence of probe for *β-actin* gene was BT297T: 5′-AAG GAG AAG CTG TGC TAC GTC GCC C-3′. The fluorescent reporter dye, a 6-carboxy-fluorescent (FAM), was located at the 5′-end of the probes and the quencher 6-carboxy-tetramethyl-thodamine (TAMRA) was located at the 3′-end.

Standard curve. Recombinant plasmids of *ob* and *β-actin* were used for plotting standard curves, respectively. These standard curves were generated by using fivefold serial dilution triplicates ranging from 10^7 to 10^3. The copy numbers of these plasmids were calculated using the following equation [23]:

1-μg of 1000 bp DNA = 9.1×10^{11} molecule.

Real-time RT-PCR amplification. Total RNA was isolated, treated by DNase I, and identified the integrity according to the corresponding methods of cDNA cloning mentioned above. Two micrograms of total RNA was transcribed using reverse transcription (RT) reagents (Promega, USA) in a 25 μl reaction volume containing 1 × M-MLV buffer, 0.5 μg random primer, 0.5 mM dNTPs, 25 U Rnasin, and 200 U Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT). The reaction was carried out at 37 °C for 1 h, and the reverse transcriptase was inactivated at 95 °C for 5 min. PCR amplification was carried out at a final 50 μl volume, containing 1 × Ex *Taq* buffer, 2 mM $MgCl_2$, 200 μM dNTP, 5 μl of cDNA or diluted plasmids, 10 pmol of each primer, 5 pmol of Taqman probe, and 1.25 U TaKaRa Ex *Taq* (TaKaRa Biotechnology Co., Ltd). Each PCR amplification was performed in triplicate wells, using the following conditions: 2 min at 95 °C, followed by 45 cycles consisting of 5 s at 95 °C and 30 s at 60 °C. The fluorescence signals were read and collected at 60 °C for each cycle. The real-time PCR was carried out on iCycler iQ™ Real-Time PCR Detection System. (Bio-Rad, USA).

Relative quantification of leptin expression. The quantities of pika leptin mRNA were normalized with *β-actin* mRNA to compensate for variations in input RNA amounts. Normalization was carried out by dividing the average value of *ob* by the average value of *β-actin* in each tissue.

Analysis of data. Data were analyzed with the statistical program SPSS 10.0. Independent sample *t* test was used for testing the data of body mass index (BMI, g/cm^2) and ob gene expression. The sample size for each site was $n = 10$.

Results

Cloning and sequence analysis of leptin cDNA of plateau pika

To obtain the full-length cDNA of plateau pika leptin, six fragments, which formed a 70–100 bp overlapping in each two neighboring sequences, were cloned by turns. The six fragments were shown as follows: OCLEP (348 bp), OCLEPA (820 bp), OCLEPB (559 bp), OCLEPC (869 bp), OC5R (339 bp), and OC3R (889 bp). Then these fragments were spliced into a 3015-bp sequence. The sequence was submitted and obtained a GenBank Accession No. DQ268537. The full-length leptin cDNA included a coding sequence of 504 bp, 5′ untranslated region (UTR) of 65 bp, and 3′UTR of 2446 bp. The open reading frame (ORF) initiated with an ATG codon at nucleotide position 66 and terminated with a TGA stop codon at nucleotide position 569. The 3′UTR contained a polyadenylation signal (AAAAT) located 13 bases upstream from the poly(A) tail. The coding sequence of pika leptin cDNA shared 83%, 82%, 81%, 81%, 81%, and 79% nucleotide sequence homology to that of pig, dog, cow, cat, human, and mouse, respectively. The entire sequence of pika leptin cDNA is shown in Fig. 2. The deduced amino acid sequence of plateau pika leptin cDNA was composed of 167 amino acids and encoded an apparent signal peptide sequence of 21 amino acids with signal cleavage site between Ala-21 and Val-22. Thus, the mature excreted protein has a predicted molecular weight of 16.086 kDa and a pI of 6.3. The result of multiple sequence alignment indicated that the mature pika leptin was 70–72% identical to that of other mammals (pig, dog, cow, mouse, rat, and human). The result of multiple sequence alignment is shown in Fig. 3. Motifs which were predicted in deduced amino acid sequence of pika leptin contained one N-glycosylation site, five Casein kinase II phosphorylation sites, one protein kinase C phosphorylation site, and one ATP synthase α and β-subunits signature site. The pika leptin protein was estimated to comprise four helixes in secondary structure. There were two cysteine residues at 117 and 167, which can form a highly conservative disulfide bond. Three-dimensional (3D) structural modeling predicted high similarity of tertiary structure between pika and human leptin (Fig. 4).

Expression analysis of ob gene in plateau pikas from different altitudes

Comparison of body mass index (BMI)

Body mass index (BMI) was significantly different between the two sites. The mean BMI at Haibei (3200 m) and Guoluo (3900 m) were 0.0423 ± 0.00241 and 0.0469 ± 0.0040 (mean ± SD), respectively, which showed a significant decrease from Guoluo to Haibei ($t = -3.106$, $p = 0.006$) Fig. 5A.

Analysis of real-time RT-PCR

Plasmid clones for target genes were used for plotting standard curves for leptin and *β-actin*. These standard curves were generated by using fivefold serial dilution triplicates ranging from 10^7 to 10^3 input copies/μl. Standard curve equations and linear regression coefficients were calculated automatically by the analysis software in iCycler iQ™ Real-Time PCR Detection System. The standard curve equation for *ob* was $Y = -3.516X + 40.735$, the correlation coefficient was 0.996. The standard curve equation for *β-actin* was $Y = -3.451X + 43.299$, the correlation coefficient was 0.991.

Comparison of ob gene expression in different altitudes

cDNA was obtained from white adipose tissues at abdominal subcutaneous of plateau pikas in two sites. The quantification assay for real-time PCR was performed in triplicate to evaluate reproducibility. Obtained copy numbers were divided by *β-actin* expression levels to normalize results (Table 1). Expression value of *ob* gene was significantly different between the two sites. The mean expression value of *ob* gene at Haibei and Guoluo was 0.43 ± 0.06494 and 0.60 ± 0.0887 (mean ± SD), respectively, which showed a significant decrease from Guoluo to Haibei ($t = -5.053$, $p = 0.000$) Fig. 5B.

Discussion

All organisms face and respond to variation in a variety of environmental stresses. In evolution, an organism must overcome new adverse conditions and establish new unique metabolic reaction to a particular environmental stress and eventually reach stable status in adaptation to their environment [14]. Phenotypic variation is determined by genotypic changes. Ecological diversity and stress can promote genetic diversity. The ecological-genetic pattern is existing in nature, because different genotypes display varying adaptation in changeable environments and stresses. Therefore, genome–phenome organization in nature is related with abiotic and environmental diversity and stress, and is strictly structured [24]. The sites of the present study have the typical cold and hypoxia plateau environment with continental monsoon type climate, and long, cold winters, and short, cool summers. Winds in the areas are frequent and harsh, especially during winter and spring. The range of daily temperature change is great. The average altitude of the sites was more than 3000 m. Therefore, harsh climate conditions on Qinghai-Tibet Plateau may have profound effects on animal survival. In evolution, autochthons must form their own adaptive strategy to

Fig. 2. Plateau pika leptin cDNA and deduced amino acid sequence. The predicted motifs and signal peptide sequence are shaded by grey background with name abbreviation under them. Boxed amino acid sequences indicate the motif site of ATP synthase α and β-subunits signature. The start codon ATG and the stop codon TGA are underlined. The asterisk in amino acid sequence indicates the stop codon. The GenBank accession number of plateau pika leptin gene is DQ268537. The abbreviation of motifs is presented as follows: CK-2, casein kinase II phosphorylation site; PKC, protein kinase C phosphorylation site; N-glycosylation, N-glycosylation site.

unique plateau environment. All these diversities of phenotype are attributed to genetic diversity of stress response genes.

The cDNA cloned from plateau pika white adipose tissue contained a 504 bp open-reading frame encoding a protein of 21 amino acids of N-terminal signal peptide and a mature peptide of 146 amino acids. Since the deduced amino acid sequence was highly homologous to leptin of other species, this was concluded to be plateau pika leptin. Two cystein residues (Cys 117 and Cys 167) forming an intramolecular disulfide bond were also conserved in pika leptin at the same position of other species. The predicted secondary and tertiary structure of pika leptin was showed to be highly similar to that of human leptin. Besides, we discovered that there were pika-specific residue substitutions in twenty sites, which were highly conserved in the corresponding sites of other species compared. This genetic diversity in pika leptin may be the results of ecological adaptation of

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pika        MRCGPLCQLLWLWPCLLCVQAVSIWKVRDDTKTLIKTILTRISDISHTHAVSSKQRITGL 60
pig         MRCGPLCRFLWLWPYLSYVEAVPIWRVQDDTKTLIKTIVTRISDISHMQSVSSKQRVTGL 60
dog         MRCGPLCRFLWLWPYLSCVEAVPIRKVQDDTKTLIKTIVARINDISHTQSVSSKQRVAGL 60
cow         MRCGPLYRFLWLWPYLSYVEAVPICKVQDDTKTLIKTIVTRINDISHTQSVSSKQRVTGL 60
mouse       MCWRPLCRFLWLWSYLSYVQAVPIQKVQDDTKTLIKTIVTRINDISHTQSVSAKQRVTGL 60
rat         MCWRPLCRFLWLWSYLSYVQAVPIHKVQDDTKTLIKTIVTRINDISHTQSVSARQRVTGL 60
human       MHWGTLCGFLWLWPYLFYVQAVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGL 60

pika        DFIPALHPNLSLSKMDQTLVLYKHILTSLPSRNVVQIANDLENLRDLLHLVAASQGCPPP 120
pig         DFIPGLHPVLSLSKMDQTLAIYQQILTSLPSRNVIQISNDLENLRDLLHLLASSKSCPLP 120
dog         DFIPGLQPVLSLSRMDQTLAIYQQILNSLHSRNVVQISNDLENLRDLLHLLASSKSCPLP 120
cow         DFIPGLHPLLSLSKMDQTLAIYQQILTSLPSRNVVQISNDLENLRDLLHLLAASKSCPLP 120
mouse       DFIPGLHPILSLSKMDQTLAVYQQVLTSLPSQNVLQIANDLENLRDLLHLLAFSKSCSLP 120
rat         DFIPGLHPILSLSKMDQTLAVYQQILTSLPSQNVLQIAHDLENLRDLLHLLAFSKSCSLP 120
human       DFIPGLHPILTLSKMDQTLAVYQQILTSMPSRNVIQISNDLENLRDLLHVLAFSKSCHLP 120
                                                                    *

pika        RASDLESLNSLESILEASLYSTEVVALSRLQGSLHEMLQQLDIGPGC 167
pig         QARALETLESLGGVLEASLYSTEVVALSRLQGALQDMLRQLDLSPGC 167
dog         RARGLETFESLGGVLEASLYSTEVVALNRLQAALQDMLRRLDLSPGC 167
cow         QVRALESLESLGVVLEASLYSTEVVALSRLQGSLQDMLRQLDLSPGC 167
mouse       QTSGLQKPESLDGVLEASLYSTEVVALSRLQGSLQDILQQLDVSPEC 167
rat         QTRGLQKPESLDGVLEASLYSTEVVALSRLQGSLQDILQQLDLSPEC 167
human       WASGLETLDSLGGVLEASGYSTEVVALSRLQGSLQDMLWQLDLSPGC 167
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Fig. 3. Multiple sequence alignment of plateau pika leptin with those of human (GenBank Accession No. NM_000230), cow (GenBank Accession No. BT020625), pig (GenBank Accession No. NM_213840), dog (GenBank Accession No. NM_001003070), rat (GenBank Accession No. NM_013076), and mouse (GenBank Accession No. NM_008493). The same amino acids between them were shaded by grey background. The numericals at the right column are the total numbers of amino acids of leptin protein of the selected species. Two cystein residues at 117 and 167 are indicated by asterisks.

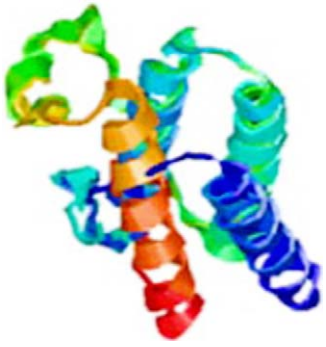

Fig. 4. Ribbon diagram shows the tertiary structure of plateau pika leptin. Secondary and tertiary protein structures were modeled using the ProMod program at the SWISS-MODEL automated protein modeling server, based upon human leptin (1AX8.pdv) Protein Data Bank Structure file.

plateau pikas living in harsh plateau environment. What effects on the function of pika leptin by this genetic diversity need us further to study.

The two sites (Haibei and Guoluo) in the present study have the typical cold and hypoxia plateau climate mentioned above. Annual rainfall largely distributes from May to September, accounting for 80% of total rainfall. The annual average growth period of herbage is about 110–130 days in the year. There is no absolute frost-free period during the year. Alpine meadow and alpine shrub are two main vegetation types. The obvious differences in the two sites are altitude and ambient temperature. The altitudes at the two sites are 3200 m (Haibei) and 3900 m (Guoluo), and the annual average temperature is −1.7 °C (Haibei) and −2.6 °C (Guoluo), respectively. Therefore, the differences in altitude and temperature in the two sites are the most important inhibiting factors having profound effects on mechanism of plateau pika in adaptation to plateau environment.

Energy expenditure contains two categories of thermogenesis: obligatory and facultative. Of the two, the former is essential for the life of all cells of the body. The largest component of obligatory thermogenesis is basal or resting metabolic rate (BMR or RMR) and diet-induced thermogenesis. Facultative thermogenesis, which can be rapidly switched on or off, occurs mainly in skeletal muscle and brown adipose tissue (BAT) when endothermic organisms are in a cold environment. Shivering thermogenesis takes place in muscle, and non-shivering thermogenesis (NST) occurs in BAT. In many small mammals BAT is heavily innervated by sympathetic nerves and is responsible for a major portion of thermogenesis during cold exposure. [25]. Thermogenesis of small mammals is determined by several factors including climate, food habit, body size,

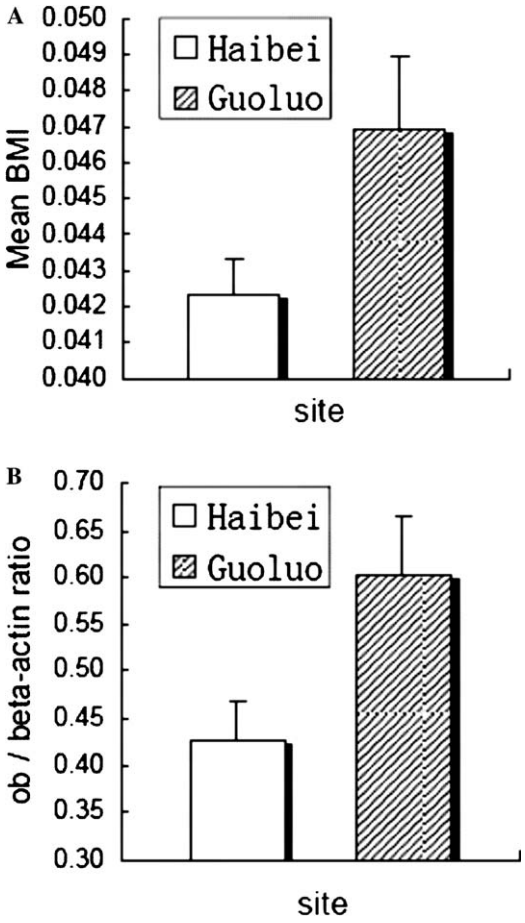

Fig. 5. Comparison of body mass index (BMI) (A) and leptin expression (B) between two sites (Haibei and Guoluo). The ob mRNA expression was determined using quantitative real-time PCR and the values were normalized relative to *β-actin* mRNA. All results are expressed as means ± SD (n=10); t- test P<0.005.

and climate and body size are the two most important among these factors. Climate condition can affect the temperature gradient between the animal body and its environment, which determines the energy expenditure in thermoregulation. During cold exposure or cold acclimate, RMR, NST, and oxygen consumption are enhanced [19]. Some researches have proved that in the long-term biological evolution, plateau pika has become a high hypoxia and low temperature tolerant mammal, and has a markedly high resting metabolic rate (RMR), non-shivering thermogenesis (NST), and high ratio of oxygen utilization to cope with the cold and hypoxia on plateau [19–21].

One of the most important functions of leptin is regulation of energy metabolism. Leptin can decrease food intake, increase body temperature, metabolic rate, and physical activity [26]. Some researches found significant positive correlations between total energy expenditure (TEE) and physical activity level (PAL) and plasma leptin concentrations [27]. Leptin increases energy expenditure through increased sympathetic activation of UCP1 gene expression in BAT, UCP2 expression in WAT, and UCP3 expression in skeletal muscle [28–30]. Leptin expression is regulated by multiple factors. Under oxygen deficiency leptin expression is increased by acting on the HIF-1-responsive element located at −116 in the leptin promoter by hypoxia-induced factor 1 (HIF-1) [10,11]. Therefore, we hypothesized that leptin, as a stress response gene to plateau cold environment, may play important roles for plateau animals in adaptation to harsh plateau environment.

In the present study, we compared body mass index of plateau pikas between two altitudes (3200 and 3900 m) and discovered that there was significant difference between two sites with larger body mass in higher altitude than in lower higher. There were no previous reports about body mass of plateau pikas in different altitudes. In the present study, we first revealed this ecological phenomenon in plateau pikas. Adipose tissue is considered as a site of lipid storage and fuel pool. In ecological adaptation of plateau pika, larger fat storage in Guoluo than in Haibei indicated that energy requirement in Guoluo is higher than that in Haibei and that the requirement of energy is increased with increasing altitude. Comparison of *ob* gene expression in the two altitudes showed that the expression level of pika *ob* gene in higher altitude and cold climate (Guoluo) is significantly higher than that in lower altitude (Haibei). The pattern of *ob* expression in different altitudes is of importantly ecological significance for plateau pika to survive in harsh plateau environment and also confirms our previous hypothesis that leptin is sensitive to plateau environment and plays an important role for plateau pika in adaptation to harsh plateau environment. With the increase in altitude, hypoxia aggravates and ambient temperature decreases, and temperature gradient between body of pika and ambient environment is increased. Plateau pika is a small, non-hibernation, diurnal mammal and never stores food, even in winter. To survive in high altitude, plateau pika must increase energy expenditure to maintain normal body temperature, physical activity, and metabolism [31]. Therefore, our results in the present study revealed the molecular mechanism of energy regulation of plateau pikas in different altitudes, that is, with the elevation in altitude, the increase in energy expenditure is performed by the gradually increased leptin expression, thus meeting high energy requirement of plateau pikas in high altitude regions.

In conclusion, in this study we reported the cloning of full-length leptin cDNA of plateau pika, sequence characteristics and compared the body mass and *ob* gene expression of plateau pikas from two altitude regions (3200 and 3900 m).To our knowledge, this is the first cloning of leptin cDNA from a natural plateau species. We first revealed the relation between harsh plateau environment and genetic diversity in stress response gene in native animals of Qinghai-Tibet Plateau. Pika leptin was 70–72% homologous to that of other species and was of similar structural characteristics with other species. The pika-specific genetic diversity in leptin sequence occurred at twenty sites. This suggested that the variation in pika leptin sequence may

Table 1
Character of sample and results of real-time PCR quantification

Site	No.	Weight (g)	Length (cm)	BMI[a]	Leptin		β-Actin		Normalized value
					Copy number	Log SQ[b]	Copy number	Log SQ	
Haibei	1	134	17.5	0.0438	(5.08 ± 2.850)E + 02	2.657	(9.21 ± 3.100)E + 07	7.945	0.33
	2	126	18.2	0.0380	(9.04 ± 1.690)E + 03	3.951	(7.94 ± 2.180)E + 06	6.889	0.57
	3	130	17.8	0.0410	(8.05 ± 2.600)E + 03	3.889	(2.02 ± 0.136)E + 08	8.305	0.47
	4	138	18.0	0.0426	(2.19 ± 0.455)E + 03	3.334	(1.01 ± 0.091)E + 08	8.005	0.42
	5	128	17.5	0.0418	(1.08 ± 0.471)E + 03	3.003	(3.64 ± 0.647)E + 07	7.556	0.40
	6	132	17.8	0.0417	(1.90 ± 0.945)E + 03	3.234	(3.85 ± 0.335)E + 07	7.585	0.43
	7	156	18.9	0.0437	(1.49 ± 0.592)E + 03	3.148	(1.45 ± 0.303)E + 07	7.156	0.44
	8	136	17.8	0.0429	(3.36 ± 3.370)E + 03	3.525	(3.67 ± 0.059)E + 08	8.565	0.41
	9	136	18.3	0.0406	(2.84 ± 1.280)E + 02	2.452	(8.64 ± 1.470)E + 06	8.821	0.36
	10	143	17.4	0.0472	(2.51 ± 1.831)E + 03	3.399	(4.40 ± 1.672)E + 07	7.641	0.45
Guoluo	1	175	17.7	0.0559	(2.15 ± 0.205)E + 05	5.331	(1.40 ± 0.155)E + 07	7.143	0.75
	2	174	19.1	0.0477	(1.76 ± 0.603)E + 03	3.227	(1.58 ± 0.202)E + 07	7.196	0.45
	3	160	18.1	0.0488	(2.88 ± 0.226)E + 04	4.459	(5.23 ± 0.534)E + 07	7.717	0.58
	4	171	19.6	0.0445	(1.32 ± 0.323)E + 05	5.113	(2.74 ± 0.402)E + 07	7.434	0.69
	5	155	19.6	0.0403	(1.11 ± 0.177)E + 05	5.043	(5.52 ± 1.490)E + 07	7.731	0.65
	6	172	19.6	0.0448	(8.94 ± 0.737)E + 04	4.951	(2.13 ± 0.446)E + 07	7.322	0.68
	7	179	19.4	0.0475	(1.86 ± 0.287)E + 04	4.267	(1.97 ± 0.263)E + 07	7.291	0.59
	8	171	19.3	0.0459	(5.07 ± 2.180)E + 03	3.703	(1.14 ± 1.090)E + 07	7.056	0.52
	9	178	19.2	0.0483	(1.33 ± 0.586)E + 04	4.123	(1.86 ± 0.133)E + 07	7.266	0.57
	10	173	19.5	0.0455	(1.49 ± 0.705)E + 04	4.173	(3.04 ± 0.665)E + 07	7.481	0.56

[a] BMI, body mass index.
[b] Log SQ, logarithm of copy number.

be the result of ecological selection of plateau pikas living in harsh plateau environment and may be having effects on functional diversity of plateau pika leptin. Besides, we first revealed the important phenomena that with the increase in altitude, there was larger fat store and higher level of ob gene expression in plateau pikas. The results are of important ecological significance for plateau pika to survive in harsh plateau environment. We first revealed one of the molecular mechanisms of energy regulation of plateau pikas in adaptation to environments of different altitudes, that is, the increase of energy expenditure of plateau pikas in different altitudes was performed by the following pathway: the gradient increase in leptin expression as initial driver upregulates UCP1 gene expression in BAT, UCP2 expression in WAT, and UCP3 expression in skeletal muscle, which increase energy expenditure to maintain normal body temperature, growth, and reproduction, and then further reinforce the tolerant capacity of cold and hypoxia. Therefore, we conclude that leptin, as an environmental stress response gene, is sensitive to cold and hypoxia plateau environment and maybe plays important roles in pikas' ecological adaptation.

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References

[1] Y. Zhang, R. Proenca, M. Maffei, M. Barone, L. Leopold, J.M. Friedman, Positional cloning of the mouse obese gene and its human homologue, Nature 372 (1994) 425–432.
[2] R.S. Ahima, J.S. Flier, Adipose tissue as an endocrine organ, Trends Endocrinol. Metab. 11 (2000) 327–332.
[3] B.S. Hamilton, D. Paglia, A.Y. Kwan, M. Dietel, Increased obese mRNA expression in omental fat cells from massively obese humans, Nat. Med. 1 (1995) 953–956.
[4] M. Maffei, J. Halaas, E. Ravussin, R.E. Pratley, G.H. Lee, Y. Zhang, H. Fei, S. Kim, R. Lallone, S. Ranganathan, P.A. Kern, J.M. Friedman, Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects, Nat. Med. 1 (1995) 1155–1161.
[5] R.C. Frederich, A. Hamann, S. Anderson, B. Lollmann, B.B. Lowell, J.S. Flier, Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action, Nat. Med. 1 (1995) 311–1314.
[6] R. Saladin, P. De Vos, M. Guerre-Millo, A. Leturque, J. Girard, B. Staels, J. Auwerx, Transient increase in obese gene expression after food intake or insulin administration, Nature 377 (1995) 527–529.
[7] G. Boden, X. Chen, M. Mozzoli, I. Ryan, Effect of fasting on serum leptin in normal human subjects, J. Clin. Endocrinol. Metab. 81 (1996) 3419–3423.
[8] M. Rosenbaum, M. Nicolson, J. Hirsch, S.B. Heymsfield, D. Gallagher, F. Chu, R.L. Leibel, Effects of gender, body composition and menopause on plasma concentration of leptin, J. Clin. Endocrinol. Metab. 81 (1996) 3424–3427.
[9] W.F. Blum, P. Englaro, S. Hanitsch, A. Juul, N.T. Hertel, J. Muller, N.E. Skakkebaek, M.L. Heiman, M. Birkett, A.M. Attanasio, W. Kiess, W. Rascher, Plasma leptin levels in healthy children and adolescents: dependence on body mass index, fat mass, gender, pubertal stage, and testosterone, J. Clin. Endocrinol. Metab. 82 (1997) 2904–2910.

[10] A. Grosfeld, V. Zilberfarb, S. Turban, J. Andre, M. Guerre-Millo, T. Issad, Hypoxia increase leptin expression in human PAZ6 adipose cells, Diabetologia 45 (2002) 527–530.
[11] A. Grosfeld, J. Andre, S. Hauguel-De Mouzon, E. Berra, J. Pouyssegur, M. Guerre-Millo, Hypoxia-inducible factor 1 transactivates the human leptin gene promoter, J. Biol. Chem. 277 (2002) 42953–42957.
[12] R. Peino, V. Pineiro, O. Gualillo, C. Menendez, J. Brenlla, X. Casabiell, C. Dieguez, F.F. Casanueva, Cold exposure inhibits leptin secretion in vitro by a direct non-specific action on adipose tissue, Eur. J. Endocrinol. 142 (2000) 195–199.
[13] P. Trayhurn, J.S. Duncan, D.V. Rayner, Acute cold-induced suppression of Ob (obese) gene expression in white adipose tissue of mice: mediation by the sympathetic nervous system, Biochem. J. 311 (1996) 729–733.
[14] K.B. Storey, Stress-induced gene expression in freeze tolerant and anoxia tolerant vertebrates, in: K.B. Storey (Ed.), Environmental stress and gene regulation, BIOS Scientific Publishers, Oxford, 1999, pp. 1–23.
[15] A.T. Smith, H.J. Smith, X.G. Wang, X. Yin, J. Liang, Social behavior of the steppe-dwelling black-lipped pika, Natl. Geogr. Res. 2 (1986) 57–74.
[16] A.T. Smith, X.G. Wang, Social relationships of adult black-lipped pikas (*Ochotona curzoniae*), J. Mammal. 72 (1991) 231–247.
[17] A.T. Smith, Patterns of pika(genus *Ochotona*) life history variation, in: M.S. Boyce (Ed.), Evolution of Life Histories: Theory and Patterns from Mammals, Yale University Press, New Haven, Connecticut, 1988, pp. 233–256.
[18] A.T. Smith, J.M. Foggin, The plateau pika (*Ochotona curzoniae*) is a keystone species for biodiversity on the Tibetan plateau, Anim. Conserv. 2 (1999) 235–240.
[19] Q.F. Li, R.Y. Sun, C.X. Huang, Z.K. Wang, X.T. Liu, J.J. Hon, J.S. Liu, L.Q. Cai, N. Li, S.Z. Zhang, Y. Wang, Cold adaptive thermogenesis in small mammals from different geographical zones of China, Comp. Biochem. Physiol. A Mol. Integr. Physiol. 129 (2001) 949–961.
[20] J.Z. Du, Q.F. Li, Effects of simulated hypoxic acclimation on organism, organ and hematology in *Ochotona curoniae* and rats, Acta Theriol. Sin. 2 (1982) 35–42 (in Chinese with English summary).
[21] J.Z. Du, Q.F. Li, X.G. Chen, Effect of simulated altitude on liver of *Ochotona curzoniae* and rats, Acta Zoolog. Sin. 171 (1984) 201–203 (in Chinese with English summary).
[22] Q.J. Wang, H.L. Shi, Z.C. Jing, C.T. Wang, F.G. Wang, Recovery and benefit analysis of ecology on degraded natural grassland of the source region of yangze and yellow rivers, Pratacult. Sci. 21 (2004) 37–41.
[23] L. Overbergh, D. Valckx, M. Waer, C. Mathieu, Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR, Cytokine 11 (1999) 305–312.
[24] E. Nevo, Evolution of genome–phenome diversity under environmental stress, Proc. Natl. Acad. Sci. USA 98 (2001) 6233–6240.
[25] G. Argyropoulos, M.E. Harper, Molecular biology of thermoregulation Invited review: uncoupling proteins and thermoregulation, J. Appl. Physiol. 92 (2002) 2187–2198.
[26] M.A. Pelleymounter, M.J. Cullen, M.B. Baker, R. Hecht, D. Winters, T. Boone, F. Collins, Effects of the obese gene product on body weight regulation in ob/ob mice, Science 269 (1995) 540–543.
[27] A.D. Salbe, M. Nicolson, E. Ravussin, Total energy expenditure and the level of physical activity correlate with plasma leptin concentrations in five-year-old children, J. Clin. Invest. 99 (1997) 592–595.
[28] P.J. Scarpace, M. Matheny, B.H. Pollock, N. Tumer, Leptin increases uncoupling protein expression and energy expenditure, Am. J. Physiol. 273 (1997) E226–E230.
[29] Y.T. Zhou, M. Shimabukuro, K. Koyama, Y. Lee, M.Y. Wang, F. Trieu, C.B. Newgard, R.H. Unger, Induction by leptin of uncoupling protein-2 and enzymes of fatty acid oxidation, Proc. Natl. Acad. Sci. USA 94 (1997) 6386–6390.
[30] Q. Liu, C. Bai, F. Chen, R.P. Wang, T. Macdonald, M.C. Gu, Q. Zhang, A. Morsy, C.T. Caskey, Uncoupling protein-3: a muscle-specific gene upregulated by leptin in ob/ob mice, Gene 207 (1998) 1–7.
[31] J.P. Su, J.K. Liu, Over winter of small herbivorous mammals inhabiting alpine area, Acta Theriol. Sin. 20 (2000) 186–192 (in Chinese with English summary).